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Radiation Induced Bystander Effects in Human Lymphoblastoid Cells

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Abstract

It is well accepted that cells, in response to radiation, may release transmissible factors. These transmissible factors, clastogenic factors, have been reported to induce genomic instability in cells that have not been directly exposed to radiation. We hypothesize that this observed bystander effect might be a consequence of cellular interactions via secretory proteins released by the irradiated cells to affect the non-irradiated cells and initiate a systemic stress response to deal with the exposure.

TK6 cells, a human lymphoblastoid cell line with a stable karyotype (47 chromosomes) and a functional p53 protein, were chosen as the surrogate for determining the stress response activation. Non-irradiated TK6 cells were co-cultured with 1 Gy γ-irradiated and non-irradiated TK6 cells in trans-wells, where the cells were kept separate but the culture media was free to diffuse across the membrane. Microarray analysis 8 hours post co-culturing monitored the gene expression changes and the dicentric assay was used to evaluate cytogenetic aberrations.

Our findings show that this research model is an effective method of demonstrating the bystander effect using the dicentric assay and γ-irradiated cells. From the cytogenetic results it is evident that a bystander effect can be seen; although chromosomal aberrations are more frequent in the irradiated samples compared to the bystander samples, the numbers seen in the bystander samples are significantly greater than those in the controls. From the microarray data, a number of possible protein biomarkers have been identified. These results, taken together, provide a foundation for our future work of identifying systemic protein biomarkers of radiation exposure.

Résumé

Lorsque des cellules sont exposées à la radiation, elles peuvent libérer des facteurs transmissibles. Ces facteurs transmissibles, appelés facteurs clastogènes, peuvent produire une instabilité génomique chez les cellules qui n'ont pas été exposées directement à la radiation. Nous formulons l'hypothèse que l'effet "bystander" observé peut être causé par les interactions cellulaires via les protéines sécrétoires libérées par les cellules irradiées en agissant sur les cellules non-irradiées et en enclenchant une réaction de stress systémique pour répondre à cette radioexposition.

Les cellules TK6, une lignée de cellules lymphoblastoïdes humaines avec un caryotype stable (47 chromosomes) et une protéine p53 fonctionnelle, ont été sélectionnées comme substitut pour déterminer l'activation de la réaction de stress. Des cellules TK6 non-irradiées ont été cultivées dans des plaques Transwell microporeuses avec des cellules TK6 irradiées avec 1 Gy de rayons γ, ou avec des cellules TK6 non-irradiées; ces plaques microporeuses maintenaient les cellules séparées mais le milieu de culture était libre de diffuser à travers la membrane. L'analyse par microréseaux effectuée 8 heures après cette coculture a permis de déceler des changements dans l'expression génétique, et l'analyse de chromosomes dicentriques a été utilisée afin d'évaluer les aberrations cytogénétiques.

Nous avons constaté que le modèle utilisé s'est avéré efficace pour démontrer l'effet "bystander" en utilisant l'analyse de chromosomes dicentriques et les cellules irradiées. Les résultats cytogénétiques démontrent clairement un effet "bystander"; même si les aberrations chromosomiques sont plus fréquentes chez les échantillons irradiés que chez les échantillons "bystander", les résultats obtenus chez les échantillons "bystander" sont de façon significative plus élevés que chez les contrôles. L'analyse de microréseaux a permis l'identification de protéines comme biomarqueurs potentiels. Tous ces résultats mis ensemble nous donneront une base pour une recherche future dans l'identification de biomarqueurs systémiques suite à la radioexposition.

Executive summary

Introduction

The Radiation Biology team of the Radiological Analysis and Defence group at DRDC Ottawa is working on developing improved biological dosimetry methods that will be used in support of physical dosimetry for identification of radiation-compromised individuals. Our laboratories are equipped with full molecular biology, cytogenetic and cell culture capabilities, and offer a suite of sources capable of delivering radiations of different qualities and amounts, as the experiments mandate.

It is well accepted that cells, in response to radiation exposure, may release certain transmissible factors. These transmissible factors have been reported to induce genomic instability in cells that have not been directly exposed to radiation. This phenomenon was first demonstrated in the plasma of persons who had been irradiated accidentally or therapeutically. It was also observed in A-bomb survivors, where the transmissible factors persisted for many years after irradiation. More recently, Emerit *et al.* reported the presence of these factors in the plasma of workers and of children exposed as a consequence of the Chernobyl reactor accident.

We hypothesized that this observed bystander effect might be a consequence of cellular interactions via secretory proteins such as cytokines released by the irradiated cells to affect the non-irradiated cells and initiate a systemic stress response to deal with the exposure.

Our group undertook the following experiments to: 1) show that we could establish an effective research model to study this bystander effect, and 2) identify possible biomarkers of radiation exposure for future development of an immunochemistry-based assay for identifying radiation exposed individuals.

Results

Our findings show that we were able to create an effective research model using trans-well cell culture plates, where the cell cultures are kept separate but the cell culture media can freely diffuse across a membrane. Our trans-well model was able to demonstrate the described bystander effect. This was evident by the chromosome damage of both the irradiated and bystander cells that we were able to visualize using the dicentric assay. Although it was evident that a bystander effect could be seen, the effect of radiation-specific chromosome damage was, as expected, greater in the irradiated samples compared to the bystander samples. Also, the numbers of damaged cells seen in the bystander samples are significantly greater than those in the non-irradiated controls. From our gene expression data, a number of possible protein biomarkers have been identified that could be indicative of radiation exposure and individual health risks.

Significance & Future Work

These results, taken together, provide a foundation for our future work of identifying systemic protein biomarkers of radiation exposure. This work includes plans to develop immunosorbent assays specific for the subset of the identified radiation specific markers. Future collaborative research may also lead to identification of systemic markers that could be indicative of biological or chemical exposures. It is anticipated that some of these markers will be common to all three stressors;

however, it is also hoped that due to the difference in gene activation by the three agents that there may also be some differences in systemic responses. It is these expected differences that may lead to specificity of agent identification.

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Sommaire

Introduction

L'équipe de Radiobiologie du groupe d'Anaylse et défense radiologiques à RDDC Ottawa travaille à développer des méthodes de dosimétrie biologique qui seront utilisées en liaison avec la dosimétrie physique pour l'identification d'individus compromis par la radiation. Nos laboratoires possèdent l'instrumentation nécessaire à la biologie moléculaire, la cytogénétique et la culture de cellules, et offrent une panoplie de sources capables de livrer des rayonnements de diverses qualités et quantités.

Lorsque des cellules sont exposées à la radiation, elles peuvent libérer des facteurs transmissibles. Ces facteurs transmissibles peuvent produire une instabilité génomique chez les cellules qui n'ont pas été exposées directement à la radiation. Ce phénomène a été observé la première fois dans le plasma de personnes irradiées accidentellement ou à la suite d'une radiothérapie. Il a aussi été observé chez les survivants de la bombe A, où les facteurs transmissibles ont persisté pendant plusieurs années après la radioexposition. Plus récemment, Emerit et al. ont remarqué la présence de ces facteurs dans le plasma de travailleurs et d'enfants exposés à la suite de l'accident du réacteur de Chernobyl.

Nous avons formulé l'hypothèse que l'effet "bystander" observé pouvait être une conséquence d'interactions cellulaires via des protéines sécrétoires comme les cytokines libérées par les cellules irradiées en agissant sur les cellules non-irradiées et en enclenchant une réaction de stress systémique pour répondre à cette radioexposition.

Notre équipe a entrepris les expériences suivantes: 1) le développement d'un modèle de recherche efficace pour étudier l'effet "bystander", et 2) l'identification de biomarqueurs d'exposition à la radiation qui pourraient être utilisés dans le développement futur d'analyses immunochimiques pour la détection d'individus exposés.

Résultats

Nos résultats démontrent que nous avons réussi à développer un modèle de recherche efficace en utilisant des plaques de culture microporeuses qui maintenaient les cellules séparées mais qui permettaient au milieu de culture de diffuser à travers la membrane. Ce modèle a démontré l'effet "bystander" par le dommage observé lors de l'analyse de chromosomes dicentriques chez les cellules irradiées et les cellules "bystander". Malgré l'observation évidente de l'effet "bystander", l'effet de la radioexposition était, comme prévu, supérieur chez les échantillons irradiés comparé aux échantillons "bystander". De plus, le nombre de cellules endommagées observées chez les échantillons "bystander" était de façon significative supérieur comparé aux contrôles non-irradiés. Selon les données d'expression génétique, un nombre de biomarqueurs potentiels ont été identifiés, ce qui permettrait l'identification de risque pour la santé chez les individus exposés à la radiation.

Importance et Recherche Future

Tous ces résultats mis ensemble nous donneront une base pour une recherche future dans l'identification de biomarqueurs systémiques suite à une radioexposition. Cette recherche comprend le développement d'analyses de type immunoadsorbant spécifiques aux biomarqueurs de radiation. Une recherche future en collaboration pourra aussi conduire à l'identificaton de marqueurs systémiques qui seraient indicatifs d'expositions biologiques ou chimiques. Il est prévu que certains de ces marqueurs seront communs aux trois agents stressants; cependant, nous espérons qu'il y aura

des différences dans les réponses systémiques causées par des différences dans l'activation génétique. Ce sont ces différences qui pourront conduire à la spécificité dans l'identification des agents.

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1. Introduction

In the event of an exposure to radiation, particularly due to nuclear accidents or terror attacks involving a large group of people, it is necessary to quickly obtain a reasonable estimation of the absorbed dose and more importantly, evaluate the radiation-induced biological effects for making immediate medical management plans as well as to assess the long-term health risks.

Physical devices such as personal dosimeters may accurately determine the radiation exposure in an environment, but fail to reflect the levels of radiation exposures to the body nor can they provide any information on the biologically meaningful doses of radiation in an exposed individual or population. It is also unlikely that members of the general public involved in a sudden accident or a CBRN incident would be carrying personal dosimeters. Clinical symptoms following an incident are also inadequate for an accurate assessment of radiation exposures and health risks. The development of biodosimetry or biomonitoring methods offers an alternative approach not only for determining the radiation exposure levels, but also to predict potential health risks in the exposed individuals.

The need for biomonitoring of radiation exposures has been re-emphasized in view of the bystander effects of radiation. It is well accepted that cells, in response to radiation exposure, may release certain transmissible factors. These transmissible factors, clastogenic factors, have been reported to induce genomic instability in cells that have not been exposed to radiation. Clastogenic factors were first described in the plasma of persons who had been irradiated accidentally or therapeutically [1, 2]. They were also observed in A-bomb survivors, where they persisted for many years after irradiation [3]. More recently, Emerit *et al*. reported the presence of these factors in the plasma of workers and of children exposed as a consequence of the Chernobyl reactor accident [4, 5, 6, 7]. Monitoring of these transmissible factors as overall indicators of biological response in human tissues/fluids would reflect levels of radiation-induced damage regardless of the specific targets being exposed.

A number of assays have been developed for use in biodosimetry procedures [8, 9, 10]. Some of these assays can be used not only for estimating radiation exposures but also, more importantly, for predicting potential health risks. The same can also be used to screen individuals with enhanced intrinsic radio-sensitivity in both healthy subjects and patients exposed to radiation. However, each of the assays has advantages and disadvantages, with some being predictive but very expensive and labour intensive and thus applicable to only a limited number of cases, while others provide delayed information. Therefore, none of these assays individually can meet all the requirements of CBRN preparedness for health protection. For that reason, additional assays should be developed which will complement existing methods to provide new dimensions in assessing radiation exposures and health risks.

The most suitable assay would be one which (i) reflects a measurable biological effect proportional to dose (over a wide range, possibly from 0.01 to 5.0 Gy), (ii) requires easily, preferably non-invasively, collected test samples, (iii) enables data collection for a large number of affected individuals in the shortest possible time after radiation exposure (rapid and high throughput), (iv) is inexpensive, less labour-intensive and reproducible with the

possibility of rapidly transferring the detection technique to other laboratories, (v) is individual specific. Currently available assays used to detect bio-indicators of radiation exposures including hematological, immunological, membrane and body fluid biomarkers, gene mutations, chromosomal aberrations and molecular probing of gene expression profiles fail to meet all of these criteria [9, 11, 12, 13]. One example is the chromosomal aberration assay (namely dicentric) in peripheral blood cells that has been reported to be very useful in biodosimetry. However, this method, although robust and considered to be radiation-specific, involves an invasive procedure to acquire blood samples, and requires highly skilled manpower and specialized tools for cytogenetic analysis which is both time-consuming and labour-intensive. Certainly, the metaphase-based cytogenetic studies, unless automated, have limitations in providing exposure data for a large number of victims within a few days. There is an urgent need for developing alternative immunochemistry-based rapid and "individual specific" methods for detection of biomarkers in non-invasively collected samples such as body fluid like saliva.

We have therefore set out in this experiment to: 1) show that we could establish an effective research model to study this bystander effect, and 2) identify possible biomarkers of radiation exposure for future development of an immunochemistry based assay for identifying radiation exposed individuals.

2. Experiments and Equipment

2.1 Experimental Design Overview

TK6 cells, a human lymphoblastoid cell line with a stable karyotype (47 chromosomes) and a functional p53 protein, were chosen as the surrogate for determining the stress response activation of a bystander effect. The techniques we have employed include the use of trans-well cell culture plates (Figure 1). These plates are designed to keep cell cultures separate, but allow the free exchange of cell culture media, growth factors and cytokines across the membrane. This tool has allowed us to study the effects of the growth factors and cytokines released by the irradiated cells on the non-irradiated cells. By using these specially designed cell culture plates the cells themselves never come into direct contact with each other, thus any observed effects are due to the released transmissible factors.

All the experiments were completed in duplicate so that we could simultaneously study both the cytogenetic and gene expression differences. This was necessary because the two assays could not be performed using a common cell culture dish.

To analyze the cytogenetic effects of this co-culturing of irradiated and non-irradiated cells we used the dicentric assay. This assay has been accepted as the International Standard for biodosimetry providing very important information needed for assessment of radiological accidents. The expected background of chromosomal aberrations in a normal (unexposed population) is generally accepted to be in the order of one dicentric per 1000 metaphase spreads scored [14].

For the analysis of the gene expression changes, RNA was extracted 8 hours post co-culturing and microarray assays were completed. The microarrays employed were Clontech's Atlas Human Cytokine/Receptor nylon membrane arrays. These arrays include 268 representative genes from several cytokine families.

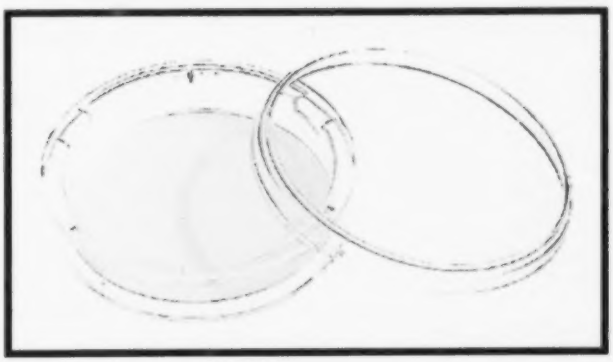

Figure 1. Costar 10 cm Trans-well Plates

The trans-well plates are designed to keep cells separate, but allow transfer of media, growth factors and cytokines across the membrane. The bottom plates used in the following experiments were 100 mm with 75 mm top plates.

2.2 Irradiation Schematic

2.2.1 Materials

- Sterile forceps
- Large beaker with bleach
- 6 trans-well dishes (Costar Cat # 3419 - 0.4 μm pore size)
- TK6 (human Lymphoblastoid) cells cultured for seven days
- ^{60}Cobalt source (GB-150-C)
- RPMI Complete Media
- 15 mL polypropylene tubes
- Pipettes
- Incubator

2.2.2 Methods

TK6 cells were cultured in complete RPMI media (RPMI-1640 with 10% FCS and 1% 200 mM L-Glutamine) for 7 days prior to conducting the experiment. The cells were maintained at concentrations between 10^5/mL and 10^6/mL.

For a single experiment 10^8 TK6 cells were required (100 mL at 10^6 cells/mL). On the day of the experiment all cells were combined and then centrifuged in 50 mL tubes at 200g for 8 min and resuspended to a concentration of 10^6 cells/mL in fresh complete RPMI media. Two 10 mL aliquots of cell suspension and two 10 mL aliquots of complete media each were irradiated with 1 Gy (100 Rad).

Three trans-well 'stacks' (top plate over bottom plate) were prepared for each assay (dicentric Assay and microarray Assay; 6 total dishes) (Figure 2). All three bottom plates contained 10 mL of 10^6 cells/mL of unirradiated TK6 cells. Top plate-1 was seeded with 9 mL of irradiated (1 Gy) complete RPMI media, Top plate-2 was seeded with 9 mL of 10^6 cells/mL of normal TK6 cells, and Top plate-3 was seeded with 9 mL of irradiated (1 Gy) 10^6 cells/mL TK6 cells.

All six plate stacks were then incubated for 8 hours at 37°C, 5% CO_2. All RNA samples were extracted eight hours post-irradiation. For the dicentric assay the cultures were set up immediately after irradiation. The total cell culture and cell fixing time for this dicentric assay was 11 hours. This entire experiment, RNA extraction and the culturing of the cells for the dicentric assay, was repeated three times.

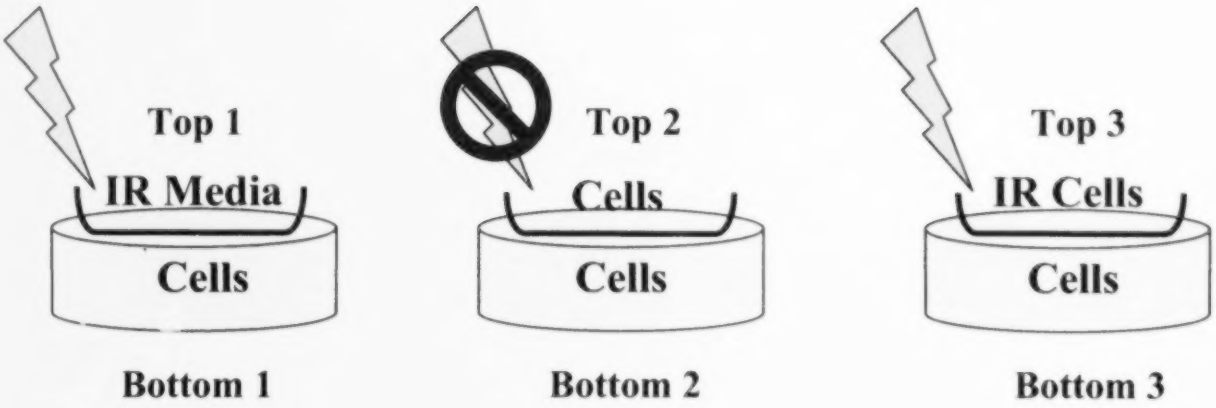

Figure 2. Experimental Design

Three trans-well 'stacks' (top plate over bottom plate) were prepared for each assay (Dicentric and Microarray). All three bottom plates contained 10 mL of 10^6 cell /mL of normal unirradiated TK6 cells. Top plate-1 was seeded with 9 mL of irradiated (1 Gy) complete RPMI media (no cells), Top plate-2 was seeded with 9 mL of 10^6 cell/mL of normal unirradiated TK6 cells, and Top plate-3 was seeded with irradiated (1 Gy) 9 mL of 10^6 cells/mL TK6 cells.

2.3 Dicentric Assay

2.3.1 Materials

- Cytochalasin B (2.5 mg/mL in DMSO)
- Methanol
- Acetic Acid
- Colcemid
- Hypotonic Solution (Potassium chloride 0.075 M)
- Centrifuge
- Incubator
- 15 mL polypropylene tubes

2.3.2 Methods

Fifty milliliter of hypotonic solution (Potassium chloride (0.56%w/v)(0.075 M) 0.56 g/100 mL) and 100 mL of fresh fix (75 mL of methanol and 25 mL of glacial acetic acid) were prepared. The irradiated media from Top plate-1 was discarded. The following steps were taken to complete the assay:

1. Eight microliters of 2.5 mg/mL stock Cytochalasin B in DMSO was added to the samples in the lower plates and 7.2 µL to the samples in the upper plates and incubated for 8 h (Cyto B concentration is 4 µL / 5 mL culture).

2. Ten microliters of 10 µg/mL Colcemid was added per 1 mL culture and incubated for 2 h.

3. Following incubation, the cells were centrifuged for 5 min at 200 x *g*, and then gently resuspended in 8 mL 37°C Hypotonic Solution.

4. The suspension was then incubated for 14 min at 37°C.

5. After the incubation the cells were gently mixed and 2 mL of fresh fixative was added. The cultures were allowed to sit for 5 min before the next step was initiated.

6. The samples were then centrifuged at 200 x *g* for 5 min, the supernatant was removed and the pellets were gently resuspended before adding, with further gentle mixing, 8 mL of fresh fixative. This step was repeated once.

7. The suspension of fixed cells was stored at -20°C until needed.

2.4 Preparation of slides from previously fixed cells (stored at –20°C)

2.4.1 Materials

- Ethanol
- Hydrochloric Acid
- Methanol
- Acetc Acid
- Microscope Slides
- Centrifuge
- 15 mL polypropylene tubes
- Glass Pasteur pipettes
- Hot Plate
- 2 - 500 mL Beakers
- 1 L cold double distilled water (ddH_2O)
- Slide Warmer
- Microscope

2.4.2 Methods

Slides were prepared from the previously fixed cells. During slide preparation, only one slide is prepared at a time. The procedure is as follows:

1. Cleaned slides were used:
   - Soaked for a few hours in 1% HCl in EtOH
   - Wiped clean
   - Stored in EtOH at –20°C
2. Fresh fixative was prepared: methanol-acetic acid (3:1).
3. The cells were centrifuged for 8 min at 200*g*.
4. Most of the supernatant was removed, leaving about 200 µL.
5. The pellet was resuspended in ~ 8 mL fixative.
6. The cells were centrifuged again for 8 min at 200*g*.
7. Most of the fixative was removed, leaving 0.5 – 1 mL (slightly cloudy appearance).
8. The slide was swished in the beaker of ice water (ddH_2O) until the water ran off smoothly.

9. The cell suspension was pipetted 2-3 times and about 15 µL was dropped onto an angled, still wet, slide.

10. The slide was flushed 3 times with a Pasteur pipette of ice-cold fixative.

11. The back of the slide was wiped.

12. The slide was held over the steaming water bath for 20 s, cell side up.

13. The slide was given one vigorous shake and the back was wiped.

14. The slide was again held over the steaming water bath for 20 s.

15. The back of the slide was wiped.

16. The slide was then labeled and moved to the slidewarmer set at about 40°C.

17. After the excess water dried (about 2 min), the slide was checked under the microscope. If the cells were too dense, more fixative was added to the cell suspension.

18. The slides were dried on the slidewarmer for at least 15 min.

2.5 Slide Staining

2.5.1 Materials

- ddH_2O
- Harleco Giemsa Stain
- Slide staining dishes
- Slidewarmer

2.5.2 Methods

1. Two staining dishes were prepared:

   - 200 mL ddH_2O + 6-8 mL Harleco Giemsa stain

   - 200 mL ddH_2O

2. The oxidation film was removed with a Kimwipe.

3. The slides were stained for 20 min.

4. After the staining incubation time the oxidation film was again removed with a Kimwipe.

5. The dish was placed in the sink with the end of the ddH_2O tubing placed into the staining dish and rinsed for 30-60 s, or until the water was clear.

6. The slides were removed to a drying rack for ~ 15 min, and then given a sharp tap to remove as much water as possible.

7. The slides were then placed on a slidewarmer and let dry overnight, covered loosely with aluminum foil.

2.6 Slide Scoring and Analysis

A minimum of 500 cells per sample were coded and blind-scored. All observed chromosome damage was recorded for each metaphase analyzed, not only dicentrics. Only complete metaphases were recorded, i.e. those with 47 or more pieces. If the cell contained unstable aberrations, only balanced spreads were accepted. For example, a spread containing a dicentric should also have an accompanying acentric fragment and still have 47 pieces. By contrast, a centric ring will also have an accompanying fragment, but the total number of objects in the cell will be 48. When high radiation doses are involved there may be more than one aberration in the spread, but the pieces should still balance. Tricentric aberrations are equivalent to two dicentrics and should have two accompanying fragments, while quadricentrics will have three fragments, and so on.

2.7 RNA Isolation

2.7.1 Materials

- QIAGEN's RNeasy RNA extraction kit
- 70% Ethanol
- 1 cc Syringes
- 20 gauge needles
- RNaseOUT Ribonuclease Inhibitor
- 15 mL tubes
- Cryovials
- Microcentrifuge
- Ribonuclease free H_2O
- β-Mercaptoethanol

2.7.2 Methods

All RNA samples were prepared eight hours post-irradiation using QIAGEN's RNeasy kit. From each of the RNA dedicated plates the TK6 cells were recovered for RNA extraction and media collection, for a total of 6 tubes. The following steps were taken to complete the assay:

1. The cells were centrifuged 5 min at 200*g* and the supernatant media was recovered for possible future testing.

2. The supernatants were frozen at – 80°C in labeled 15 mL tubes.

3. The cell pellets were loosened thoroughly by flicking the tubes, and then the appropriate volume of Buffer RLT was added (600 µL for 5 x 10^6 – 1 x 10^7 cells). To mix, the tubes were vortexed.

4. The samples were homogenized by passing the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to a 1 cc RNase-free syringe.

5. One volume (600 µL) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting.

6. Up to 700 µL of the sample was applied, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 mL collection tube. The tube was closed gently, and centrifuge for 15 s at ≥ 8,000*g* (≥10,000 rpm). The flow-through was discarded after each step.

7. Seven hundred microliters of Buffer RW1 was added to the RNeasy column. The tube was closed gently, and centrifuged for 15 s again to wash the column. The flow-through was discarded along with the collection tube.

8. The RNeasy column was transferred into a new 2 mL collection tube and then 500 µL of Buffer RPE was pipetted onto the RNeasy column. The tube was closed gently, and then centrifuged for 15 s to wash the column. The flow-through was discarded.

9. Another 500 µL of Buffer RPE was added to the RNeasy column. The tube was closed gently, and centrifuged for 2 min to dry the RNeasy silica-gel membrane. The flow-through was discarded and the tube was re-spun for an additional 1 min at full speed.

10. To elute, the RNeasy column was transferred to a new 1.5 mL collection tube and 30-50 µL RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was closed gently, and centrifuged for 1 min to elute.

11. Two microliters of RNaseOUT Ribonuclease Inhibitor was added to each extraction.

12. The RNA was stored in labeled cryovials at -80°C.

2.8 Microarray Assay

2.8.1 Materials

- Clonetech's Atlas microarray kit (contains reagents and membranes)
- Salmon testes DNA
- [α-^{32}P]dATP
- PCR Thermocycler
- Hybridization Oven

- Hybridization Bottles
- ddH_2O
- Forceps
- 15 mL polypropylene tubes
- 20X Sodium chloride/sodium citrate Buffer (SSC)
- 10% Sodium dodecylsulphate (SDS)
- Saran Wrap
- Phosphorimager (Reader and Cassettes)
- Microcentrifuge
- 0.5 mL PCR Tubes

2.8.2 Methods

Five milligrams of RNA was labeled for each membrane and the MMLV-RT enzyme was used to catalyze the reaction. The mRNA label used in these experiments was ^{32}P. All arrays were globally normalized and then compared. Only those genes with at least a two-fold mean difference were included in the results. All array results are the result of three replicate experiments. [Because this microarray method uses the radionuclide ^{32}P, all methods were conducted in our radiation biological laboratory using the approved safety guidelines.]

2.8.2.1 Prehybridization setup

1. A bottle of ExpressHyb was pre-warmed to 65°C.

2. The first hybridization bottle was filled with ddH_2O and the membrane was then placed into the bottle with the hybridization spots facing into the volume, not against the glass. The water was then poured off into the second hybridization bottle, repeating with the second membrane, and so on for all membranes.

3. Salmon testes DNA (10 mg/mL) was thawed at room temperature. For 4 membranes: 400 µL was transferred to a 0.5 mL PCR tube and heat-denatured in the PCR thermocycler at 95°C for 5 min and then chilled on ice. Seventy-five µL was transferred to each of four 12 mL aliquots of ExpressHyb. The solution was mixed vigorously until the DNA was completely dispersed into the ExpressHyb.

4. Eight milliliters of ExpressHyb + DNA suspension was transferred to each hybridization tube (1 membrane/tube). The solution was quickly and evenly distributed over the membrane, to prevent drying.

5. The membranes were pre-hybridized for at least 30 min with continuous agitation at 65°C. During the pre-hybridization, the Master Mix and labeled probes were prepared.

2.8.2.2 Probe Labeling

A Master Mix and RNA samples were prepared for the labeling reactions. For the Master Mix one extra reaction was always taken into consideration for possible pipetting errors.

1. For the Master Mix the following reagents were combined in a 0.5 mL PCR tube:

   - 10 µL 5X Reaction Buffer
   - 5 µL 10X dNTP Mix
   - 2.5 µL DTT (100 mM)
   - 17.5 µL [α- ^{32}P]dATP

2. For each reaction 4-5 µg of RNA was prepared. The volumes of RNA were normalized across samples with ddH_2O, but did not exceed 2 µL. The total volume including cDNA synthesis (CDS) primer did not exceed 3 µL. The following was combined in 4 labeled 0.5-mL PCR tubes:

   - RNA Sample
   - ddH_2O

3. One microliter of CDS Primer was added to each tube and they were mixed well by pipetting. The tubes were then spun briefly in a microcentrifuge if needed.

4. The tubes were incubated in a preheated PCR thermocycler at 70°C for 2 min.

5. The temperature was then reduced to 50°C for 2 min. During this incubation, 1 µL MMLV RT per reaction was added to the Master Mix. The solution was then mixed by pipetting and was kept at room temperature until used.

6. After 2 min at 50°C, the temperature was reduced to 42°C, and then 8 µL of Master Mix was added to each reaction tube. The RNA samples were not removed longer than necessary to add Master Mix.

7. The tubes were returned to the thermocycler.

8. The tubes were incubated in the PCR thermocycler at 42°C for 25 min.

9. The reaction was stopped by adding 1 µL of 10X Termination Mix to each tube.

2.8.2.3 Hybridizing cDNA Probes to the Atlas Membrane Arrays

At no time during the hybridization preparation were the Atlas membrane arrays allowed to cool.

1. Five microliters of Cot-1 DNA was added to each labeled probe.

2. One hundred microliters of ExpressHyb solution was added to each tube.

3. The probe was incubated in the thermocycler at 95°C for 5 - 10 min. To prevent radioactive contamination, foil was used to protect the block and lid of the thermocycler and the caps of the PCR tubes were pierced to prevent from blowing open.

4. The samples were quenched on ice.

5. The probe was added to each respective pre-hybridization solution being careful to avoid pouring the concentrated probe directly on the surface of the membrane. The two solutions were mixed by gently swirling the solution.

6. The membranes were hybridized overnight with continuous agitation at 65°C.

2.8.2.4 Washing the Membranes and Exposing

The next day the Atlas membrane arrays were washed and then placed in a cassette for exposure of the phosphorimage. In steps 3, 4, and 5 the membranes were never allowed to cool before the addition of wash solutions.

1. The hybridization oven was pre-warmed to 68°C.

2. The Wash Solutions were prepared for 4 membranes. Solutions 1 and 2 were pre-warmed to 68°C.

   - **Wash Solution 1** (2X SSC, 1 % SDS)
   - **Wash Solution 2** (0.1X SSC, 0.5% SDS)
   - **Wash Solution 3** (2X SSC)

3. The hybridization solution was carefully removed and discarded in an appropriate radioactive waste container. The hybridization tubes were rinsed briefly with ~10 mL of Wash Solution 1. The wash was then discarded as radioactive waste and replaced with ~50 mL of pre-warmed Wash Solution 1. The Atlas membrane

arrays were washed for 30 min with continuous agitation at 68°C and then the solutions were poured off into the radioactive waste. This step was repeated.

4. One 30-min wash was performed in ~80 mL of pre-warmed Wash Solution 2 with continuous agitation at 68°C.

5. One final 5 min wash was performed in ~100 mL of Wash Solution 3 at room temperature.

6. Using forceps, the Atlas membrane arrays were removed from the hybridization bottles and any excess Wash Solution was shaken off. The Atlas membranes arrays were not allowed to dry.

7. The damp Atlas membrane arrays were immediately wrapped in Saran wrap and then positioned on the phosphorimaging cassette.

8. The arrays were checked for activity using the survey meter.

9. The cassette was placed in a dark cupboard for 7 days.

10. On the seventh day the phosophorimaging screens were scanned in the phosphorimager. The images were saved for later analysis using Clontech's Atlas software.

3. Summation

3.1 Results

3.1.1 Cytogenetic (Dicentric Assay) Results

A minimum of 500 (average 549) cells were coded and blind-scored for each sample. See Table 1 for the complete data set. The cytogenetic results from the dicentric assay reveal that the irradiated cells had the highest frequency of dicentrics or rings (0.016) per cell as well as the highest frequency of total chromosome damage (0.257) (Figure 3A and B, respectivly). The bystander cells had a frequency of 0.007 dicentrics or rings per cell and the frequency of total damage was 0.044 (Figure 3A and B). Both the Normal Top and Normal Bottom non-irradiated controls (Table 1 and Figure 2, Stack 2) and irradiated media Negative Control (Table 1 and Figure 2, Stack 1) had no dicentric chromosomes or rings. These samples did, however, display some non-radiation specific chromosome damage (Figure 3B and D). Only the irradiated cells and the bystander cells displayed Tri-Quadraradials (TRQs) (Figure 3E). There were a total of four TRQs in the irradiated cells and one in the bystander cells. Figure 4 shows examples of cytogentic damage found in the bystander cells.

Table 1. Cytogenetic Results (Raw Data).

D=Dicentric, AF= Acentric Fragment, TRQ=Tri-Quadraradial, R=Ring, CT=Chromatid Break, 48=one extra chromosome

Sample	Plate Code	Normal	D	D+AF	AF	TRQ	R	R+AF	CT	48	Other	Abnormal Cells	Total Damage	Total Cells
Negative Control	1 Bottom	519	0	0	10	0	0	0	1	7	2	20	20	**539**
Normal Top	2 Top	550	0	0	4	0	0	0	6	5	1	16	16	**566**
Normal Bottom	2 Bottom	511	0	0	2	0	0	0	1	3	1	7	7	**518**
Irradiated	3 Top	447	4	5	89	4	0	0	15	6	24	126	147	**573**
Bystander	3 Bottom	527	1	2	10	1	0	1	4	3	2	22	24	**549**

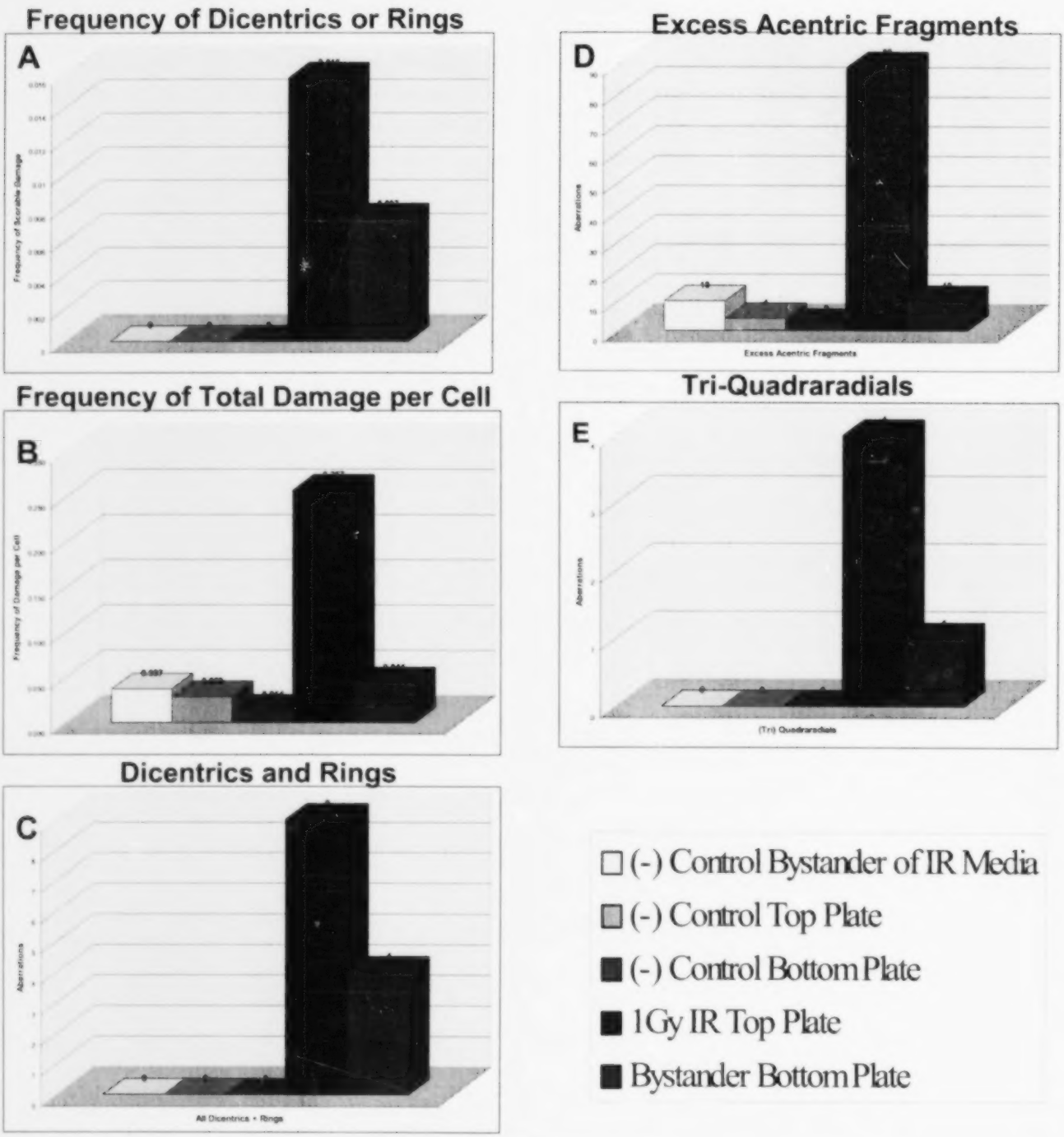

Figure 3. Cytogenetic Data

A minimum of 500 (average 549) cells were coded and blind-scored for each sample. Panel A and B show the frequency of damage, and panels C-E show the number of specific aberrations in each sample.

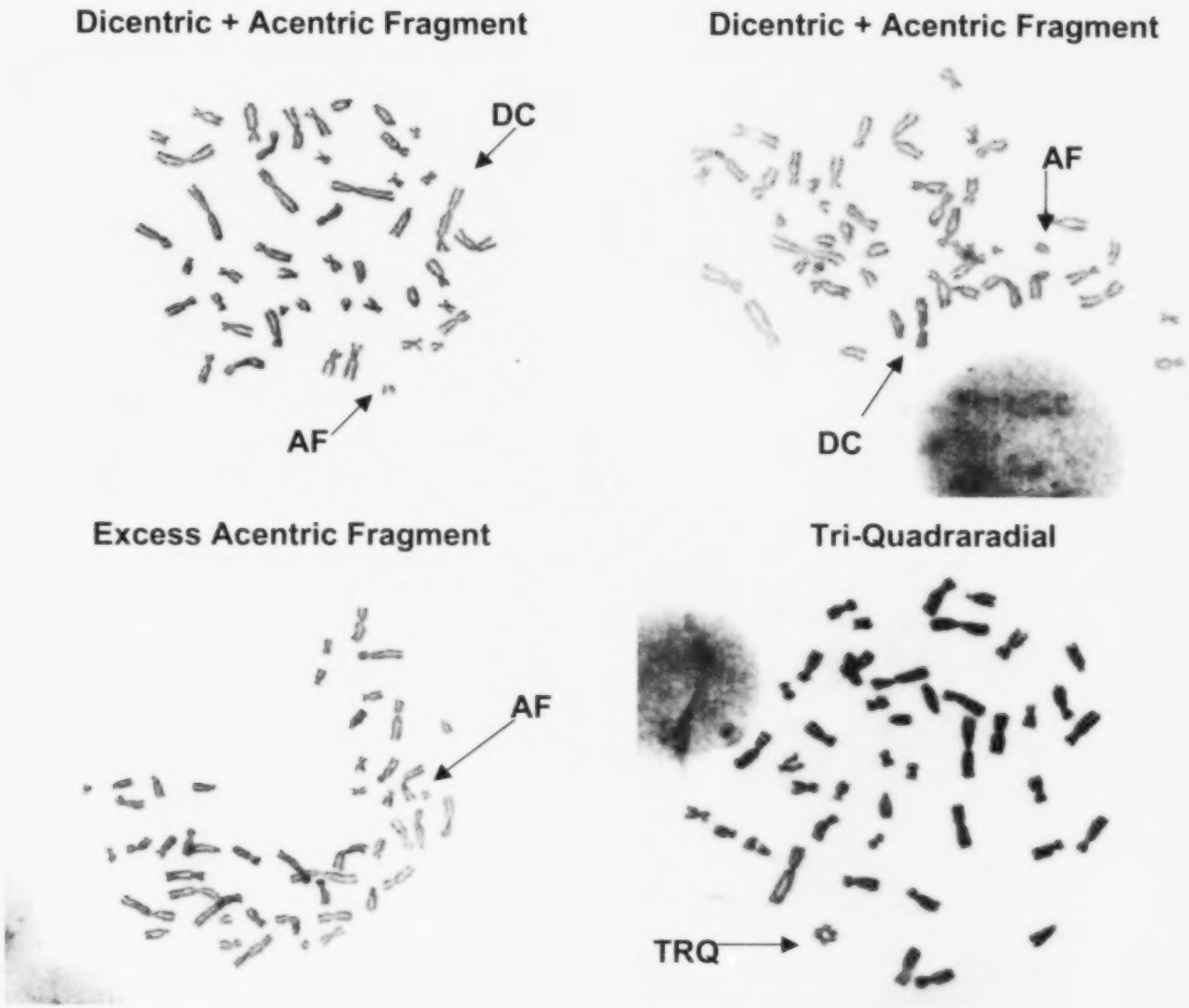

Figure 4. Cytogenetic Damage in the Bystander Cells

3.1.2 Microarray Results

The gene expression data of the non-irradiated normal cells compared to the negative control cells (exposed to irradiated cell culture media only) showed that 16 genes were differentially expressed by at least 2-fold. Suprisingly, all 16 genes were upregulated in the normal cells compared to the bystander cells (Figure 5). When the same normal non-irradiated cells were compared to the bystander cells, ten genes were differentially expressed (Figure 6). Similarly, when the irradiated cells were compared to the normal cells, ten genes were also expressed differentially, although they were not the same ten (Figure 7). Finally, when the irradiated cells were compared to the bystander cells, nine genes were differentially expressed (Figure 8).

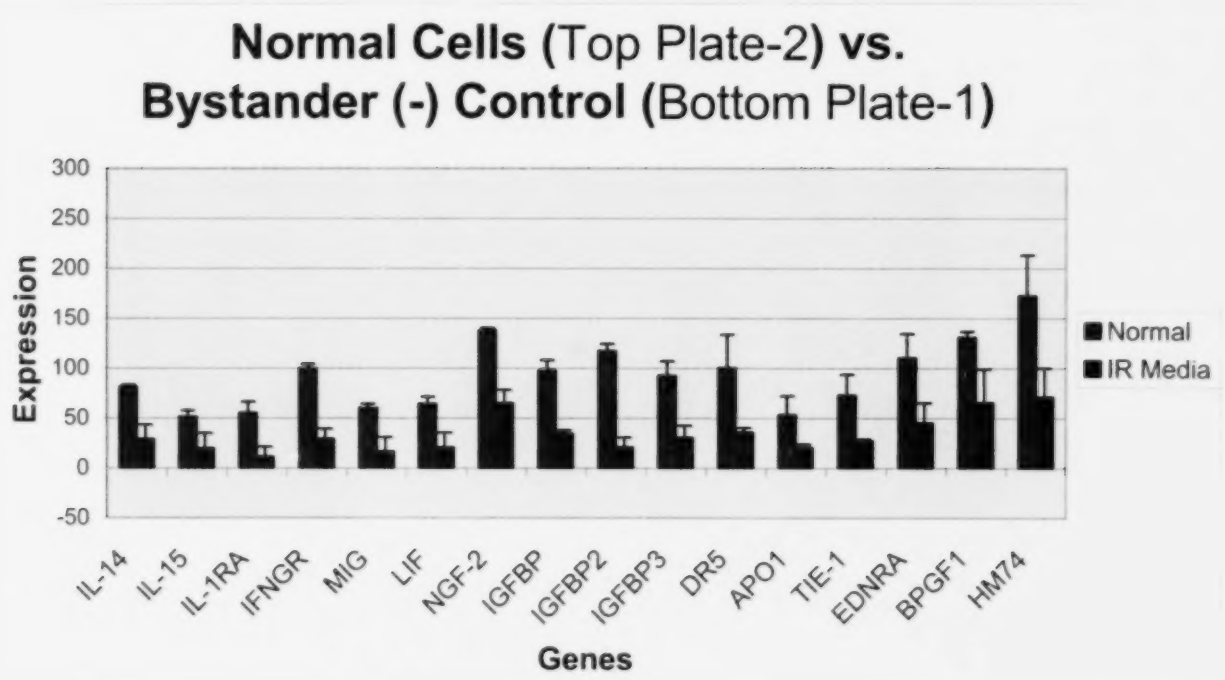

Figure 5. Microarray Data Comparing the Normal and Negative Control Cells

All RNA samples were prepared eight hours post-irradiation using QIAGEN's RNeasy kit. The samples were treated with RNaseOUT and stored at –80° C until ready for use. Five micrograms of RNA was labeled for each membrane and the MMLV-RT enzyme was used to catalyze the reaction. The label used in these experiment was ^{32}P. All arrays were globally normalized and then compared. Only those genes with at least a two-fold mean difference were included in the results. All array results are the result of three replicate experiments.

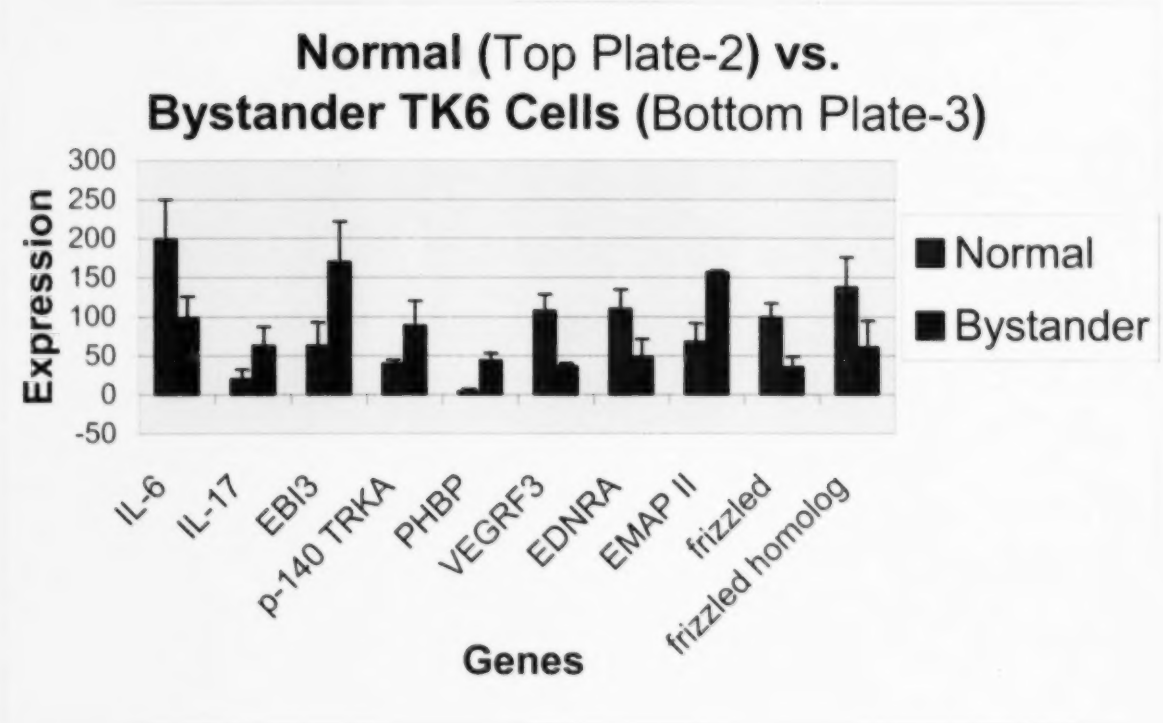

Figure 6. Microarray Data Comparing the Normal and Bystander Cells

All RNA samples were prepared eight hours post-irradiation using QIAGEN's RNeasy kit. The samples were treated with RNaseOUT and stored at –80° C until ready for use. Five micrograms of RNA was labeled for each membrane and the MMLV-RT enzyme was used to catalyze the reaction. The label used in these experiment was ^{32}P. All arrays were globally normalized and then compared. Only those genes with at least a two-fold mean difference were included in the results. All array results are the result of three replicate experiments.

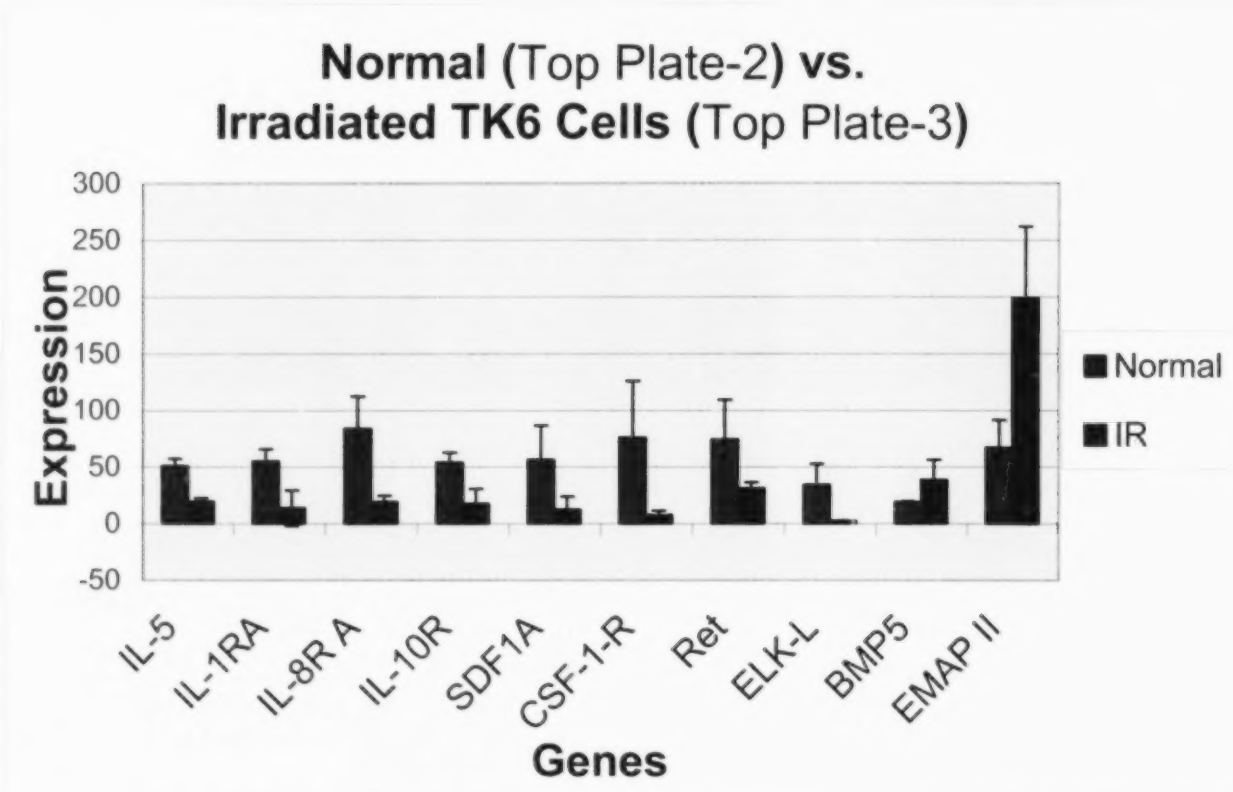

Figure 7. Microarray Data Comparing Normal and Irradiated Cells

All RNA samples were prepared eight hours post-irradiation using QIAGEN's RNeasy kit. The samples were treated with RNaseOUT and stored at –80° C until ready for use. Five micrograms of RNA was labeled for each membrane and the MMLV-RT enzyme was used to catalyze the reaction. The label used in these experiment was ^{32}P. All arrays were globally normalized and then compared. Only those genes with at least a two-fold mean difference were included in the results. All array results are the result of three replicate experiments.

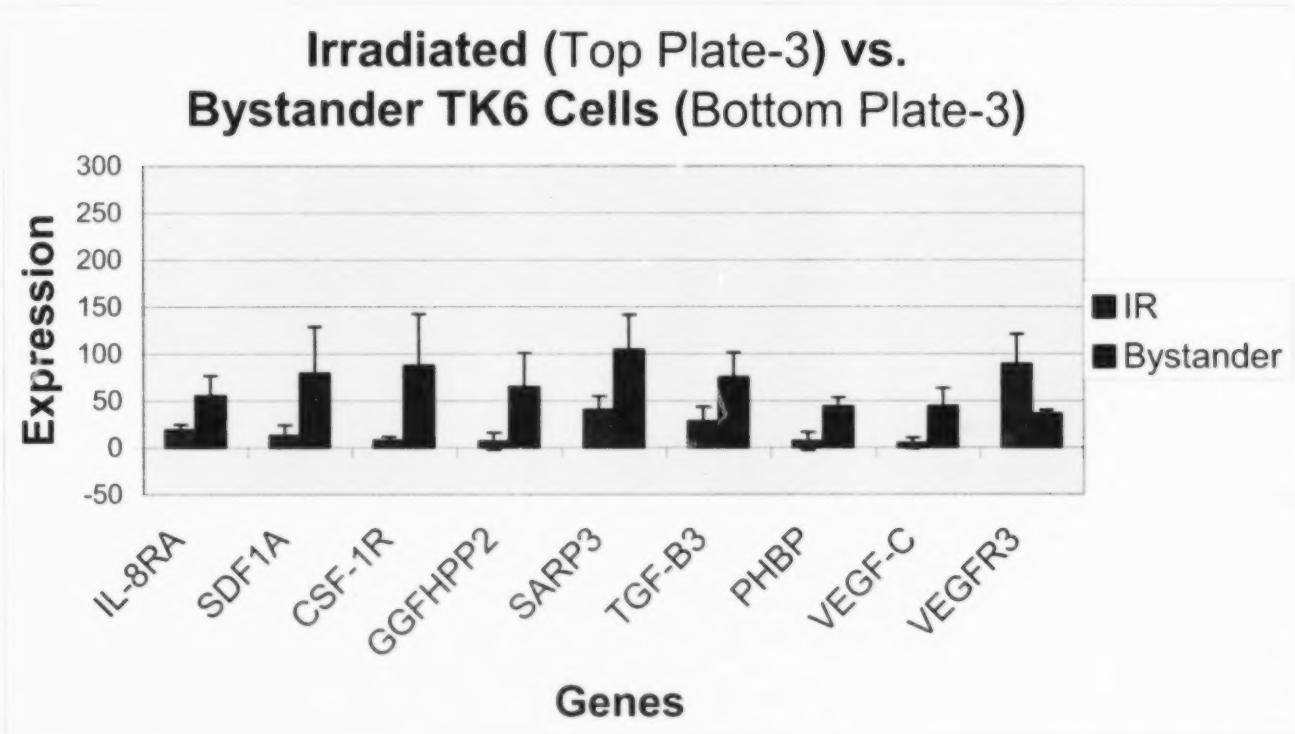

Figure 8. Microarray Data Comparing Irradiated and Bystander Cells

All RNA samples were prepared eight hours post-irradiation using QIAGEN's RNeasy kit. The samples were treated with RNaseOUT and stored at –80° C until ready for use. Five micrograms of RNA was labeled for each membrane and the MMLV-RT enzyme was used to catalyze the reaction. The label used in these experiment was ^{32}P. All arrays were globally normalized and then compared. Only those genes with at least a two-fold mean difference were included in the results. All array results are the result of three replicate experiments.

3.2 Discussion

Our first objective was to show that this experimental design was an effective method of demonstrating the bystander effect using the dicentric assay and γ-irradiated cells. From the dicentric (and ring) cytogenetic results it is evident that a bystander effect can be seen using this method (Figure 3A and C). Another interesting finding was the correlation of TRQs in the irradiated and bystander samples (Figure 3E). Although the effects for dicentrics and TRQs are greater in the irradiated samples compared to the bystander samples, the numbers seen in the bystander samples are significantly greater than those in the controls. This large difference, however, is not seen between the bystander negative control (irradiated media) and the bystander samples in regards to acentric fragments (Figure 3D). This may be due to the fact that acentric fragments are not radiation specific, allowing for a background of this kind of damage as well as the possibility of the presence of oxidative products in the irradiated media.

Interestingly, in the microarray data the largest number of differentially expressed genes was found between the normal TK6 cells and the bystander negative control (Figure 5). One possible reason for this would be the dilution of the growth factors since the upper well only contained irradiated media and no cells, thus accounting for a dilution factor of two in respect to cytokines or growth factors. Also interesting was that all the differentially expressed genes between the normal and the irradiated cells resulted in genes more highly expressed in the normal cells, with the exception of EMAPII, the inflammatory cytokine released under apoptotic conditions, and BMP5, a bone morphogenetic protein (Figure 7). To pair with this microarray data, we plan to further analyze secretory proteins in cell culture media samples (cell culture supernatant) using a liquid protein array system.

3.3 Conclusions

Our findings show that we were able to create an effective research model using trans-well tissue culture plates, where the cell cultures are kept separate but the cell culture media can freely diffuse across a membrane. Our trans-well model was able to demonstrate the described, bystander effect. This was evident by the chromosome damage of both the irradiated and bystander cells that we were able to visualize using the dicentric assay. Although it was evident that a bystander effect could be seen, the effect of radiation-specific chromosome damage was, as expected, greater in the irradiated samples compared to the bystander samples. Also, the numbers of damaged cells seen in the bystander samples are significantly greater than those in the non-irradiated controls. From our gene expression data, a number of possible protein biomarkers have been identified that could be indicative of radiation exposure and individual health risks. These will be used for protein profiling of stored cell culture media of irradiated cells and of future donor samples received from medically exposed individuals. This gained information will enable us to devise a panel of markers that can be used for identification of radiation exposed individuals.

These results, taken together, provide a foundation for the development of immunosorbent assays specific for the subset of the identified radiation specific markers. Future collaborative research may also lead to identification of systemic

markers that could be indicative of biological or chemical exposures. It is anticipated that some of these markers will be common to all three stressors; however, it is also hoped that due to the difference in gene activation by the three agents that there may also be some differences in systemic responses. It is these expected differences that may lead to specificity of agent identification.

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List of symbols/abbreviations/acronyms/initialisms

CBRN	Chemical Biological Radiological and Nuclear
cDNA	Complimentary DNA
CDS Primer	cDNA Synthesis Primer
ddH_2O	Double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DND	Department of National Defence
dNTP	Deoxynucleoside triphosphate
DRDC	Defence Research and Development Canada
DTT	Dithiothreitol
EtOH	Ethanol
FCS	Fetal Calf Serum
Gy	Gray
HCl	Hydrochloric Acid
MMLV-RT	Moloney murine leukemia virus reverse transcriptase
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPMI Media	Roswell Park Memorial Institute (Where the media was developed)
SAIC Canada	Science Applications International Corporation Canada
SDS	Sodium dodecylsulphate

SSC	Sodium chloride/sodium citrate (buffer)
TK6	Thymidine Kinase heterozygote cell line
TRQ	Tri-Quadraradial

Glossary

Technical term	Explanation of term
acentric	Terminal or interstitial chromosome fragment of varying size. When it is formed independently of a dicentric or centric ring chromosome aberration, it is usually referred to as an excess acentric.
antibodies	Protein (immunoglobin) produced in response to an antigenic stimulus with the capacity to bind specifically to the antigen.
biomarkers	A biochemical, such as a protein, that can be used as an indicator of a specific biological response.
cDNA	Complimentary DNA. Single-stranded DNA complementary to an RNA transcript, synthesized from it by reverse transcription *in vitro*.
Colcemid	Methylated derivative of colchicines.
colchicine	Drug isolated from the Autumn crocus that blocks microtubule assembly as a result of interfering with microtubule reassembly will block mitosis at metaphase.
centric ring	Aberrant circular chromosome resulting from the joining of two breaks on separate arms of the same chromosome (generally accompanied by an acentric fragment).
centromere	Specialized constricted region of a chromosome that appears during mitosis joining together the chromatid pair.
chromosome	46 of these structures that carry genetic information are normally contained in the human cell nucleus. During nuclear division they condense to form characteristically shaped bodies. There are; however, 47 chromosomes in the cell cultures used in these experiments.
chromosome aberrations	Any change resulting in the duplication, deletion, or rearrangement of chromosomal material.

clastogenic factors	Factor found in the blood that could cause breaks in chromosomes.
cytogenetics	The study of chromosomes, the visible carriers of DNA, the hereditary material. Cytogenetics is a fusion science due to joining of cytology (the study of cells) with genetics (the study of inherited variation).
cytokines	Polypeptides, originally defined as being released from lymphocytes and involved in maintenance of the immune system. These factors may exert effect on hematopoietic and non-hematopoietic cells.
dicentric	Chromosome aberration resulting from annealing of the centromeric pieces of two broken chromosomes (accompanied by an acentric fragment).
DNA (deoxyribonucleic acid)	A nucleic acid found in most living cells; it carries the organism's hereditary information.
dosimetry	Measurement of a radiation dose exposure.
gene	One of the biological units of heredity located on chromosomes; transmits hereditary information.
gene expression	The process of producing a protein from its DNA- and mRNA-coding sequences.
genomic instability	Abnormally high rates of genetic change occurring serially and spontaneously in descendents of the same cell-populations.
karyotype	The characterization of the chromosomal complement of an individual or a species, including number, form, and size of the chromosomes.
lymphoblastoid	Tumour cell derived from a white blood cell that produces antibodies and is an essential component of the immune system.
metaphase	The stage of cell division when chromosomes are aligned at the cell center prior to separation. A "metaphase spread" refers to the view of a cell's chromosomes in the metaphase stage on a slide.

microarray	A molecular biology process used for quantification of gene expression by utilizing DNA complementarity's as a means of recognition.
molecular biology	The branch of biology that deals with the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins, and especially with their role in cell replication and the transmission of genetic information.
nucleotide	The basic building blocks of nucleic acids. The nucleotides commonly found in DNA are (deoxy-) adenine, guanine, cytosine, and thymine. The nucleotides in RNA are adenine, guanine, cytosine and uracil.
p53	A 393 residue phosphoprotein that is a tumour suppressor gene rather than an oncogene, as it is frequently inactivated or mutated in tumours and transformed cells.
PCR	A procedure that enzymatically amplifies a DNA sequence through a repeated replication process.
plasma	The nonliving fluid component of blood within which various solutes are suspended and circulated.
RNA (ribonucleic acid)	An organic acid composed of repeating nucleotide units of adenine, guanine, cytosine, and uracil, whose ribose components are linked by phosphodiester bonds.
Tri-Quadraradials (TRQs)	A chromosome aberration in which two chromosomes have developed an affinity for each other. This aberration can present either as a tri or quadraradial structure.

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It is well accepted that cells, in response to radiation, may release transmissible factors. These transmissible factors, clastogenic factors, have been reported to induce genomic instability in cells that have not been directly exposed to radiation. We hypothesize that this observed bystander effect might be a consequence of cellular interactions via secretory proteins released by the irradiated cells to affect the non-irradiated cells and initiate a systemic stress response to deal with the exposure.

TK6 cells, a human lymphoblastoid cell line with a stable karyotype (47 chromosomes) and a functional p53 protein, were chosen as the surrogate for determining the stress response activation. Non-irradiated TK6 cells were co-cultured with 1 Gy g-irradiated and non-irradiated TK6 cells in trans-wells, where the cells were kept separate but the culture media was free to diffuse across the membrane. Microarray analysis 8 hours post co-culturing monitored the gene expression changes and the dicentric assay was used to evaluate cytogenetic aberrations.

Our findings show that this research model is an effective method of demonstrating the bystander effect using the dicentric assay and g-irradiated cells. From the cytogenetic results it is evident that a bystander effect can be seen; although chromosomal aberrations are more frequent in the irradiated samples compared to the bystander samples, the numbers seen in the bystander samples are significantly greater than those in the controls. From the microarray data, a number of possible protein biomarkers have been identified. These results, taken together, provide a foundation for our future work of identifying systemic protein biomarkers of radiation exposure.

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